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## OSMOTICALLY INDUCED COALESCENCE OF SARCOMA CELLS THROUGH INTERCELLULAR BRIDGES IN VITRO

by Clarence D. Cone, Jr., and Max Tongier, Jr.

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NATIONAL AERONAUTICS AND SPACE ADMINISTRATION

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SUMMARY

A relatively detailed description is presented of a microtechnique by which cells possessing true intercellular bridges in vitro can be induced to coalesce into single mono- and multinucleate giant cells. The technique consists in the carefully controlled perfusion of a cell field with a hypotonic solution, which causes osmotic swelling and thereby induces connected cells to coalesce through expansion of the connecting bridge. Details of the perfusion system and methodology are discussed, along with the system of time-lapse cinemicrography used for recording the coalescence process. Principal features of an alternate coalescence technique utilizing a movable micropipet are also outlined. Representative photographic sequences reproduced from time-lapse film recordings are presented to illustrate the basic nature of the coalescence phenomenon for the case of L-strain fibroblasts. An interpretation of the implications of coalescence in indicating cytoplasmic continuity of intercellular bridges, and other aspects of intercellular communication at the molecular level, is included. Finally, a brief mention is made of several additional cytological applications of the osmotic swelling procedure.

INTRODUCTION

The Langley molecular biophysics laboratory (MBL) is presently engaged in a basic research program on the mechanics of mitosis initiation in human cells. This program is intended to provide the fundamental information necessary for elucidating specific molecular mechanisms by which ionizing space radiations and other agents of space environments act to alter or arrest cell division. The resultant upset of mitotic homeostasis in the body is manifested in the major acute and latent radiation-sickness syndromes.

In the initial course of these studies, mitotically active intercellular bridge systems were observed in cultures of L-strain mouse fibroblasts. These systems are of considerable potential interest for mitotic-initiation studies since the bridges offer a convenient means for investigating the nature of the mitotic stimulus and its transmission. These time-lapse film observations of mouse sarcoma cell cultures have been described in reference 1. It was suggested therein that transmission of a mitotic stimulation through

intercellular bridges could also in principle constitute a potential means of malignant proliferation in the animal body. For such induced mitosis to be possible, however, all unit-cells of a given syncytium must be in approximately the same metabolic state regarding preparedness for mitosis. Hence, the presence of fully open bridges or channels for free molecular communication between cells would appear to be a prime requirement for establishing such metabolic synchrony and also for transmitting molecular or ionic stimuli in such a mitotically self-exciting system.

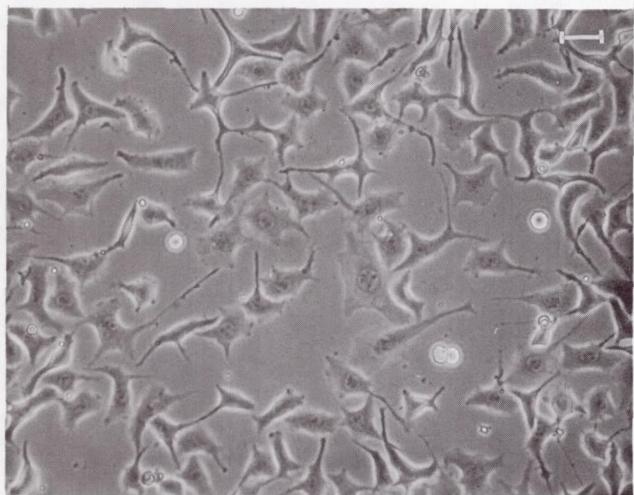
In reference 1, brief mention was made of results obtained by an osmotic swelling technique whereby bridge-connected cells in vitro were caused to coalesce into single mono- and multinucleate giant cells, thus demonstrating the continuity of the intercellular bridges. The coalescence technique itself, however, was not described. The purpose of this paper is to present the details of this technique and to describe more fully the nature of the coalescence phenomenon as recorded by high-magnification ( $\times 500$ ) time-lapse cinemicrography. The simplicity of the coalescence method makes it an especially valuable tool for preliminary investigation of bridge continuity in lieu of the more exact but much more complex and time-consuming electron-microscope examination of fixed material. In addition to its use for syncytium coalescence studies, the basic osmotic swelling technique outlined herein has found valuable application in a number of other cytological studies, a few of which are mentioned briefly in a later section of this paper.

## EXPERIMENTAL TECHNIQUES FOR INDUCING COALESCENCE

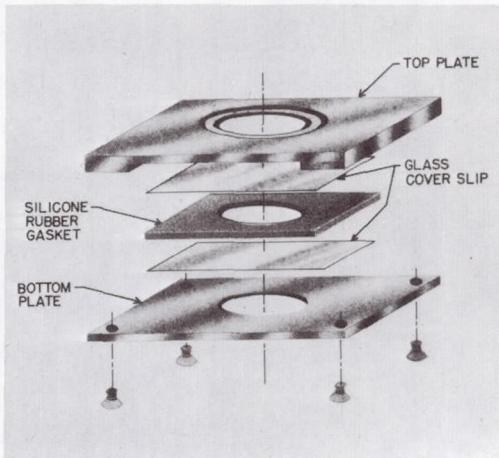
The general procedure for osmotic coalescence consists in the relatively brief exposure (2-minute pulsation) of a test field of bridge-connected cells to a hypotonic test solution. Exposure is maintained for an interval of time adequate to permit sufficient osmotic swelling for coalescence to occur. Since the cells must be swelled to a considerable extent to induce coalescence, the time of the exposure pulse and the hypotonicity of the test solution must be controlled very carefully to avoid overexpansion and rupture of the cell membrane before coalescence can take place. The entire swelling and coalescence sequence is filmed in detail using time-lapse cinemicrography.

### Test Cells and Culture Media

All experimental work covered in this report was performed with Earle's L-strain (Clone 929) of mouse fibroblasts. A brief historical background and description of the basic characteristics of this cell line are given in the appendix of reference 2. Stock cultures of these cells were maintained in Falcon plastic culture flasks with Eagle's minimum essential medium (Hank's base) supplemented with 10 percent horse serum. Figure 1 presents a micrograph of a typical monolayer culture of the L-strain in this medium.



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Figure 1.- Micrograph of field of L-strain fibroblasts illustrating characteristic intercellular bridges in monolayer cultures.  
Calibration: 50 $\mu$ .



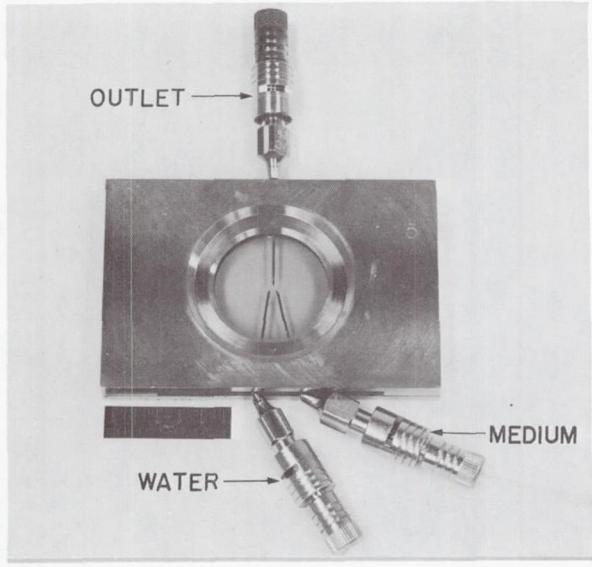
L-68-10,016  
Figure 2.- Exploded view of unassembled Rose-type MBL perfusion chamber used in coalescence studies.

#### Test Chamber and Apparatus for Osmotic Perfusion

Test chamber.- The test chamber used is illustrated in figure 2 and consists of a modified version of the basic form originally introduced by Rose (ref. 3). The essential modification consists in the provision of two precisely dimensioned end-spacers integrally machined with the top cover plate. These spacers allow uniform compression of the gasket and cover slips and thereby reduce medium leaks and cracking of the cover glasses.

The test chamber is inoculated with a suspension of cells of suitable concentration in normal culture medium and the cells are allowed to settle and attach to the cover glass. The chamber is then incubated at 37° C until a sufficiently large number of cell pairs and groups having intercellular bridges and bridge networks has formed. (See ref. 1 for a discussion of the ontogeny of bridge formation.) This incubation period may range from 8 to 24 hours, depending upon the initial number density of the settled monolayer. The optimum cell density depends upon the nature of the particular test being conducted and the optical magnification being used for recording.

Perfusion system.- To permit the attainment of sufficiently rapid and precise variation of the osmotic environment of the cells to be coalesced, treatment was restricted to a small test field in the center of the test chamber cover glass. For this purpose, two 25-gage hypodermic needles are inserted into the chamber through the silicone rubber gasket in a converging manner, as illustrated in figure 3, with the tips parallel to and



L-68-10,017  
Figure 3.- Perfusion chamber and needle system used for coalescence experiments.

Model 255-2), and containing the appropriate test solution or medium. The pump drive is adjusted for the particular syringe size being used so that a flow of approximately 0.2 ml/min is delivered by each inlet needle when the corresponding pump is in operation. By alternately activating the separate pumps for the hypotonic test solution and culture medium in proper sequence while simultaneously viewing the field under treatment, the swelling (or shrinking) rate of a given cell or cell pair can be precisely controlled.

In the preparation of the syringes, tubing, and needles for coalescence experiments, it is important to insure that all air has been removed from the perfusion system. Otherwise, air will enter with the treatment solutions, upsetting the intended flow rates and causing bubble formation which will block or obscure photographic recording of cell responses.

Perfusion by micropipet.- An alternate osmotic perfusion technique which has been quite successful in accomplishing cell coalescence consists in the use of a single micro-pipet for delivering the hypotonic test solution to the vicinity of the cell field. The experimental setup for this technique is illustrated in figure 4. In this procedure, the hypotonic solution issues continuously from the tip at a low but constant rate. By control of the proximity of the pipet tip to the test field with the use of a micromanipulator, precise regulation of the resultant or effective hypotonicity of the local medium environment of

approximately 0.5 millimeter below<sup>1</sup> the cover slip surface containing the attached cell field. These needles serve to introduce, respectively, the hypotonic test solution and the normal isotonic culture medium used for swelling control. A third 25-gage needle inserted through the opposite side of the gasket serves as an outlet for the liquid displaced by the incoming treatment solutions. The tip of the outlet needle is located approximately 2 millimeters from the inlet needle openings.

Each of the inlet needles is connected by Clay-Adams Intramedic PE-160 plastic tubing and Luer fittings to a glass syringe, mounted in a variable-drive perfusion pump (Sage Variable Speed Syringe Pump,

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<sup>1</sup>For time-lapse recordings using a conventional microscope, the test chamber is oriented with the cover slip containing the cells uppermost.

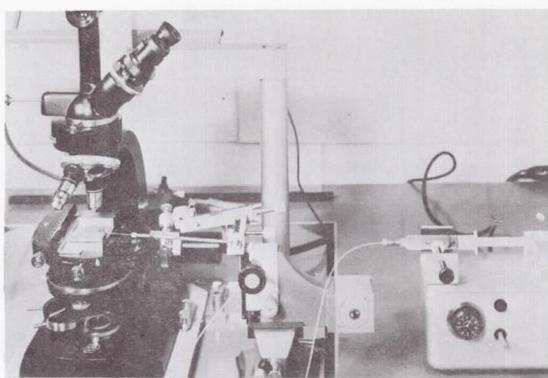
the test field can be attained, as evidenced by the close control of cell swelling which is possible. Mixing and diffusion of the inlet stream with the culture medium of the chamber results in a region of increased hypotonicity near the pipet tip, and time and degree of cell exposure to the hypotonic environment can be effectively regulated by pipet movement. A moist chamber (fig. 4) is required with this arrangement unless an inverted microscope and associated time-lapse unit are utilized for recording. However, the micropipet technique has the advantage that any preselected cells or cell groups in the entire cell field can be treated, and many coalescence tests can be run with a single culture.

#### Time-Lapse Film Recording

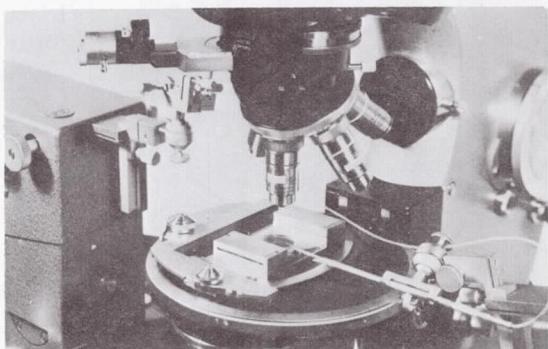
All film recordings for tests reported herein were made with a Sage Series 100 cinemicrographic apparatus (Model 088) provided with a Zeiss Standard WL research microscope using phase-contrast optics. The test chamber with attached perfusion system is mounted in the incubator of the unit, which allows maintenance of a constant temperature over the course of an experiment. Figure 5 is a photograph of the general recording setup. The Sage time-lapse unit allows recording at film rates from 1 frame per 4 minutes to 64 frames per second. Because of the relatively rapid occurrence of events in coalescence, all tests were recorded at 32 frames per minute; this rate was adequate for recording all events in sufficient detail.

#### Procedure for Osmotic Induction of Coalescence

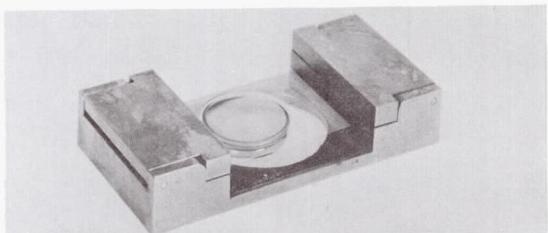
For a typical osmotic coalescence experiment, a test chamber is mounted in the microscope of the time-lapse unit and a suitable bridge-connected cell pair located in the test field; the unit is then activated and recording is commenced. The optical system of



(a) Closeup view of pipet system mounted for time-lapse recording (time-lapse incubator shield removed).

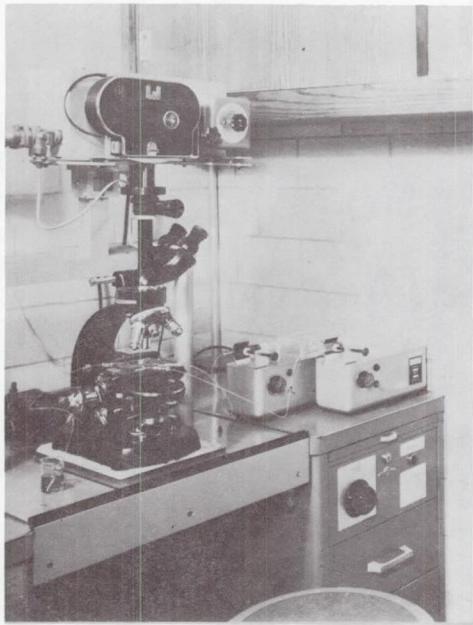


(b) Pipet system mounted in micromanipulator (photomicroscope arrangement).



(c) Closeup view of MBL moist chamber designed for micromanipulator use.

L-68-10,018  
Figure 4.- Micropipet perfusion system.



L-68-10,019  
Figure 5.- General view of setup for recording coalescence using needle perfusion system.  
Incubator cover removed.

the time-lapse unit is such that the test cell field can be observed directly during recording. The perfusion pump delivering the hypotonic solution is switched on and the subsequent swelling of the cells monitored closely to avoid too rapid a swelling rate. Use of the proper swelling rate is important since overly rapid expansion causes severe blebbing of the cell surface with attendant premature rupture of the membrane. If the swelling rate is initially observed to be excessive, the perfusion pump is switched off, whence the cell immediately commences shrinking as a result of the return of the local environment to the normal isotonic condition. The process may be repeated several times on the same cell, with sequential adjustment of the flow rate until the proper swelling rate is established. Once determined, the same pump setting is usually adequate for all subsequent coalescence experiments. For the L-strain, a flow rate of approximately 0.2 ml/min is generally adequate

and allows coalescence to occur within a period of 1 to 2 minutes, when water alone is used as the swelling agent. However, times required for coalescence often vary widely, depending upon the length and diameter of the bridge, culture conditions, and the like. Although it is desirable or necessary to use other hypotonic solutions for specific test purposes, as will be discussed subsequently, for all studies of this report water was used to effect coalescence.

To obtain ready coalescence of cell pairs, it is necessary that there be no intervening obstructions, such as other cells. Upon swelling of a bridge-connected pair, the cells are pulled together with considerable force by the tension of the bridge, and any retardation of cell movement usually results in the breakage of the connecting cytoplasmic bridge.

#### TIME-LAPSE OBSERVATIONS OF L-CELL COALESCENCE

Four sets of photographs of frames from actual time-lapse film recordings illustrating typical coalescence sequences resulting from hypotonic swelling of L-cells are presented in figures 6 to 9. The needle perfusion technique was used to obtain all observations on coalescence illustrated in this section. The particular frame set illustrated in figure 6 shows two separate cell pairs in the process of coalescing, each pair forming a single binucleate giant cell. In frame A, the cells are attached to the culturing surface

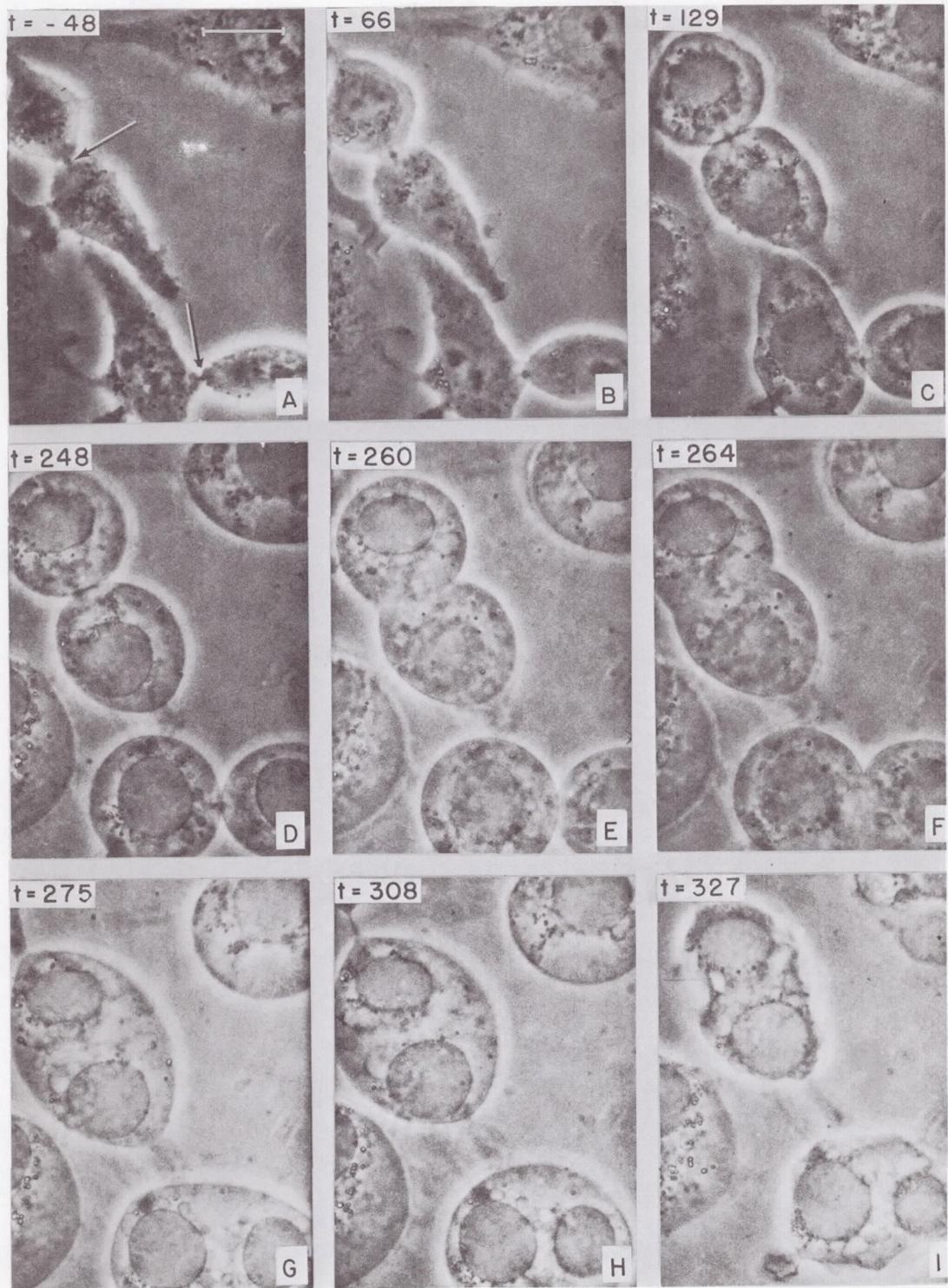


Figure 6.- Photograph sequence of time-lapse film frames illustrating coalescence process. Note simultaneous coalescence of two cell pairs in field. Numbers denote time  $t$  in seconds from commencement of hypotonic perfusion. Calibration:  $20\mu$ .

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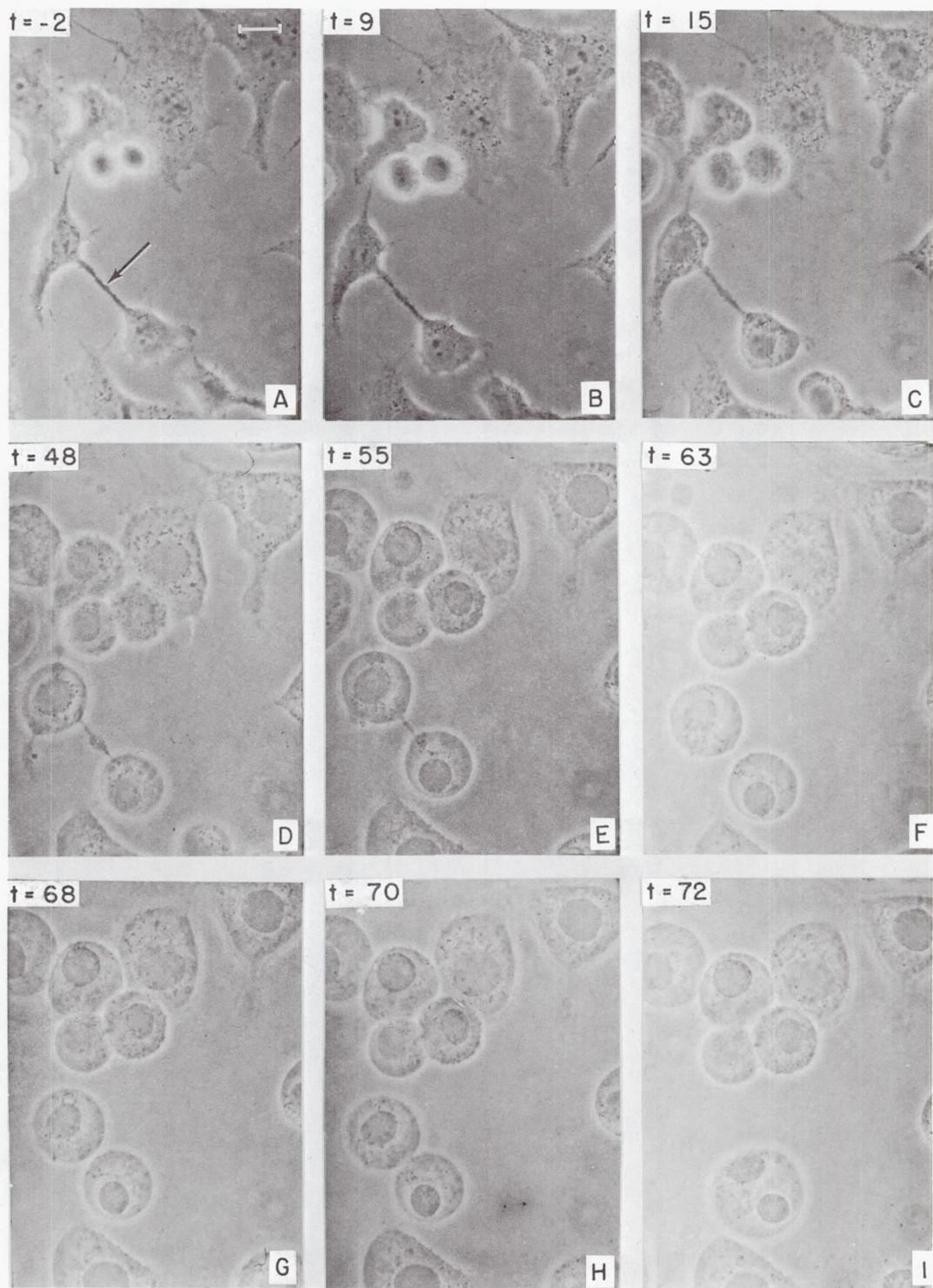
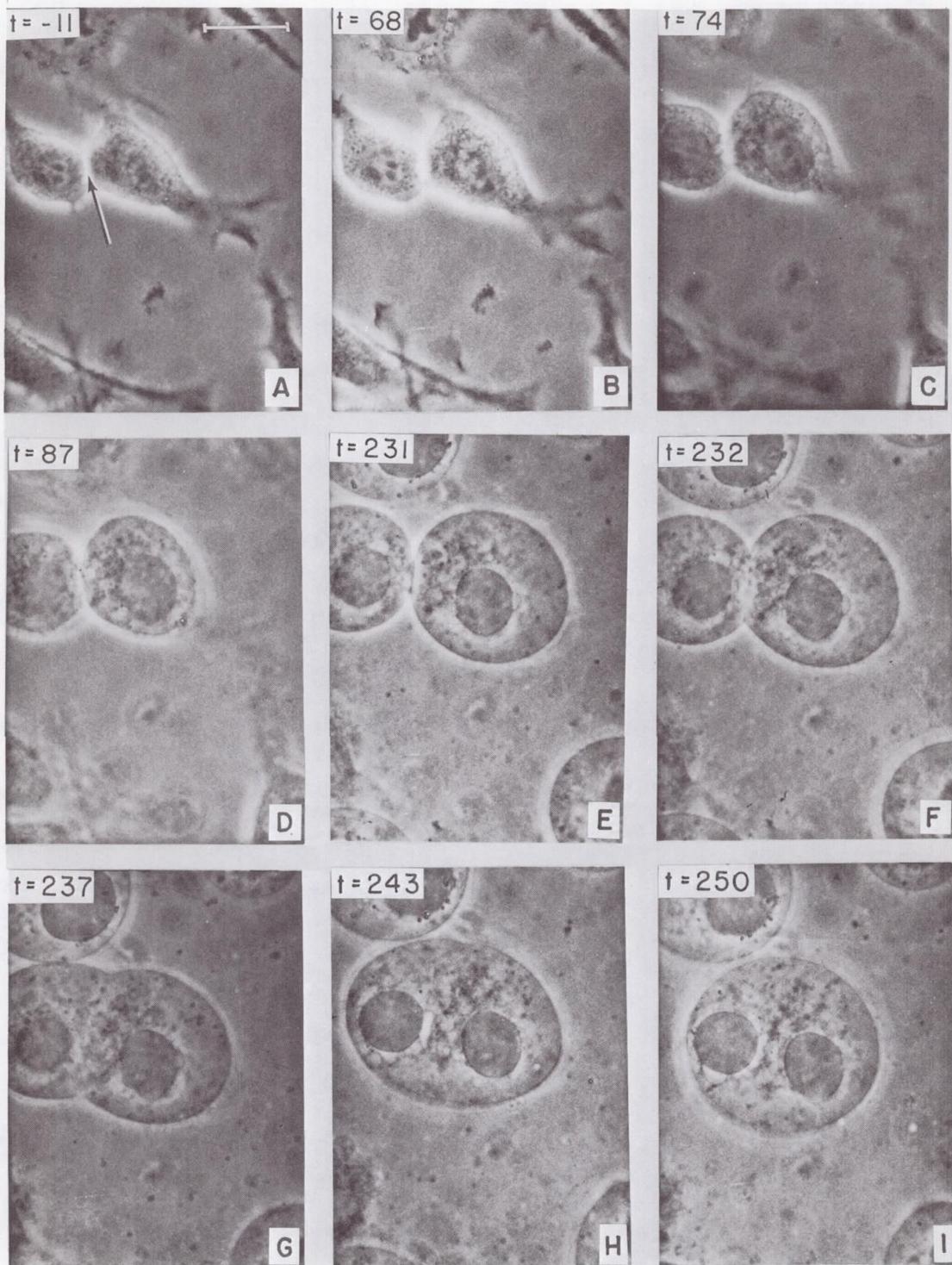
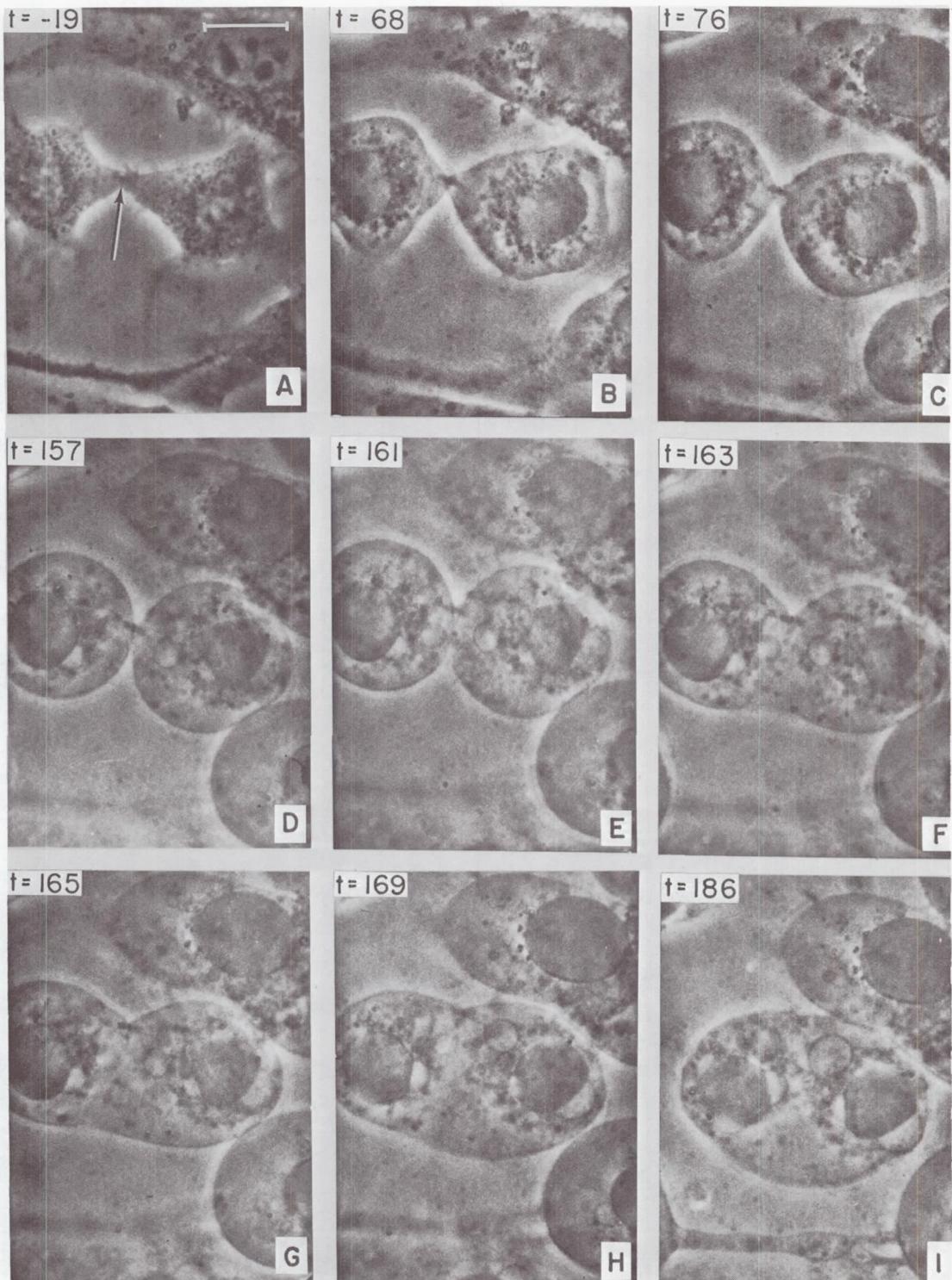


Figure 7.- Illustration of cell-pair coalescence by hypotonic swelling involving a long, thin intercellular bridge. L-68-10,021  
Numbers denote time  $t$  in seconds from commencement of hypotonic perfusion. Calibration; 20 $\mu$ .



L-68-10,022  
Figure 8.- Cell-pair coalescence by hypotonic swelling. Numbers denote time  $t$  in seconds from commencement of hypotonic perfusion. Calibration:  $20\mu$ .



L-68-10,023  
Figure 9.- Additional illustration of cell coalescence sequences from time-lapse film. Numbers denote time  $t$  in seconds from beginning of hypotonic perfusion. Calibration;  $20\mu$ .

prior to treatment. In frame B, some 66 seconds after start of hypotonic perfusion, both the cytoplasm and nuclei are beginning to swell detectably. It is a general observation in all swelling experiments that the nucleus appears to undergo a slight swelling before any clearly discernible volume increase in the cytoplasm takes place; thus a relatively high degree of "osmotic" sensitivity of the nucleus is suggested. Swelling continues in frames C and D, with the cells drawing together tightly as the bridge contracts to zero length. Swelling of the nuclei is especially pronounced in these frames, and the nucleoli, if still intact within the nuclei, are no longer distinguishable. In frame D, just before coalescence, the cells and their nuclei are essentially spherical under the internal swelling and have released from the surface except at their single points of contact. The cytoplasm has undergone considerable solation at this time because of the water influx, as is indicated by the pronounced increase in Brownian motion of the intracellular particles.

Coalescence has commenced for the upper cell pair in frame E of figure 6 and involves a sudden expansion of the cell membrane at the constriction, primarily on the left side. This sudden stretching of the membrane is quite graphic when viewed in time-lapse projection. The lower cell pair coalesces in the same manner almost simultaneously. In frame G, two large binucleate cells with fully intact nuclei have formed. Immediately after frame G, hypotonic perfusion was stopped and normal culture medium introduced into the chamber so that in frame H the cells have already shrunk by an observable amount. The nuclei, however, are still fully distended. By frame I, the cytoplasmic volume has been greatly reduced although the nuclei are still appreciably swollen. Ultimately, however, the nuclei also shrink toward their former size.

As is clearly illustrated in frame I, the shrunken cell assumes a wrinkled or puckered appearance. This wrinkling is indicative of an excess of membrane surface area for the cytoplasmic and nuclear volumes enclosed and is an expected consequence of two aspects of the coalescence process. The first is the geometrical fact that when the volumes of two small spheres (or of any geometrically similar forms) are combined to produce a single larger sphere, the combined surface area of the small spheres is larger than the total surface area of the larger sphere. Hence, following coalescence of two single spherical cells and reduction of the resultant cytoplasmic mass to its original total volume, the resultant giant cell has a considerable excess of membrane surface and is no longer spherical in form. Flattening of the cell on the culture surface will ultimately allow the cell to spread the wrinkled membrane, as the surface-to-volume ratio increases with the geometrical form change accompanying flattening. The second factor involved is the inelastic set or stretch of the membrane caused by the extensive swelling required for coalescence. It is well known that cell membranes exhibit an appreciable stretching hysteresis (refs. 4 and 5) indicating an imperfect elasticity which results in an

increase of the total membrane surface area following stretching. The wrinkling phenomenon is also observed for the nuclei; however, in cases where the nuclei do not fuse (as in fig. 6), wrinkling is due entirely to stretch hysteresis.

Despite the severe degree of membrane stretching and volume increase (cytoplasmic dilution), viable giant cells may often be obtained by coalescence provided considerable care is exercised in maintaining the proper rates for both swelling and shrinking. These cells exhibit the same characteristics as naturally formed giant cells and may live for several days, moving freely about over the culture surface. It has been found that induction of coalescence by a gradual volume increase using a series of alternate swellings and shrinkings rather than by a single continuous swelling favors the production of viable giant cells. Similarly, pulsed shrinking appears beneficial in this regard.

Coalescence of cell pairs by micropipet follows essentially the same course as illustrated in figure 6 for the fixed-needle technique. One especially valuable application of the pipet technique, however, is the production of multiple coalescence, wherein as many as five bridge-linked cells have been coalesced into a single pentanucleate giant cell. Coalescence is induced in this case by moving the pipet along the cell chain, whence sequential coalescence is obtained. The micropipet is particularly effective in producing highly localized swelling of the cytoplasmic bridges and this local expansion may contribute to the success of multiple coalescence with the technique. Because of the necessary movement of the pipet during multiple coalescence, a meaningful time-lapse recording with this technique is difficult to obtain.

The photograph set of figure 7 illustrates a coalescence of two cells connected by a rather long bridge. It is of interest to note the localized swelling of the bridge tube itself which occurs in frame D. These particular cells are being swelled rather rapidly and final coalescence occurs quickly, being complete in less than 2 seconds, as shown in frames H and I.

Two additional coalescence sequences taken from time-lapse films are illustrated in figures 8 and 9.

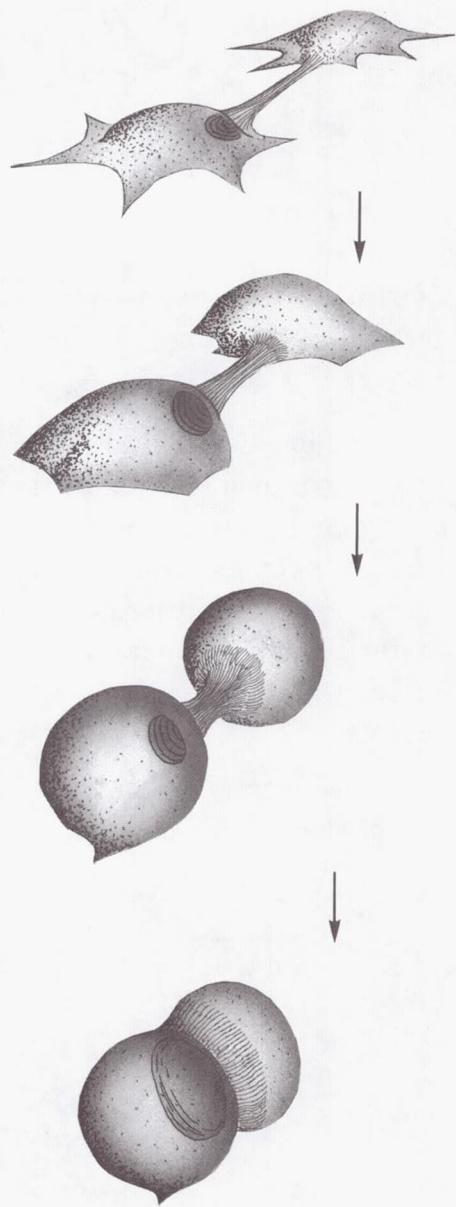
Although the coalescence sequences of figures 6 to 9 are typical and result in the formation of binucleate cells, it often happens that the nuclei of the combining cells also coalesce to produce a mononucleate giant cell. The nuclei appear to have a higher tendency to fuse in cells which are coalesced just after a previous mitosis with incomplete cytokinesis.

## IMPLICATIONS OF COALESCENCE FOR EXISTENCE OF BRIDGE CONTINUITY AND MOLECULAR TRANSPORT

### Bridge Continuity

Detailed studies of time-lapse observations of osmotic coalescence in bridge-connected cells of the L-strain give every indication that the bridges are continuous, open cytoplasmic channels and hence capable of allowing free ionic and molecular interchange between the cells. The fact that the cells do indeed readily coalesce to form a single spherical cell is taken as ample demonstration of the uninterrupted continuity and elastic structural integrity of the bridge-cell surface. The general picture of the bridge-cell pair as revealed by the coalescence films (and schematically illustrated in fig. 10) is that of a continuous cytoplasmic mass, roughly proportioned into a dumbbell form. This figure illustrates particularly clearly the view of the coalescence process as the inverse of cell division with incomplete cytokinesis (ref. 1).

The cytoplasmic continuity of the bridge is clearly indicated in time-lapse by long-term, phase-contrast films of bridge-connected cells in monolayer culture, which show the passage of particulate matter through the bridge from one cell to the other during normal growth. Similarly, in many coalescence sequences using micropipets, particulate matter can be seen moving through the bridge under the cytoplasmic flows induced by the differential pressure of the asymmetric swelling. The true cytoplasmic nature of the bridge can be demonstrated quite readily with micropipet perfusion (fig. 11). Slow movement of an ultrafine pipet, issuing pure water, along the length of the bridge results in a temporary but pronounced local swelling of the tube, producing a local bulge similar to that appearing in frame D of figure 7. Such traverses along the full length of the bridges give no indication of local constrictions, since the form of the moving



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Figure 10.- Schematic illustration of coalescence as a shape transformation of the continuous syncytial membrane surface.

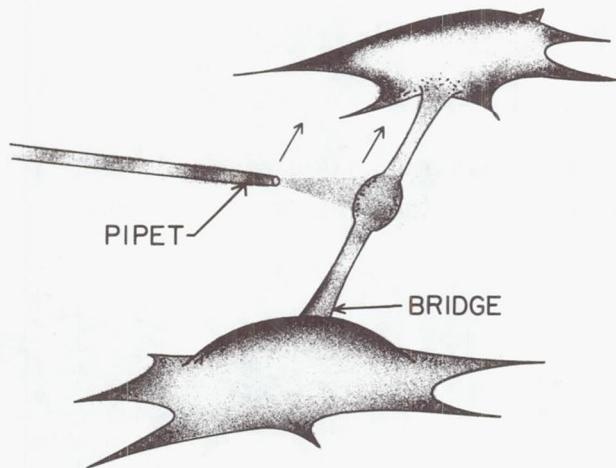


Figure 11.- Sketch illustrating local swelling of cytoplasmic bridge by micropipet perfusion.

"hypotonic bulge" is essentially constant throughout the movement. Even in cases wherein a temporary constriction of the bridge exists immediately following incomplete cytokinesis (the area of the so-called Flemming body), swelling causes the membrane to separate readily from the internal body and to expand normally. This observation is in accord with the electron-microscope data of Fawcett (ref. 6), which indicates that the "body" (at least in the case of spermatid formation) is really an annular structure with no discernible attachment to the membrane.

### Osmotic Expansion Dynamics

As is evident from the foregoing photographs of actual coalescence, the bridge itself undergoes little expansion (increase in diameter) compared with the cells, but slowly decreases in length as the cells expand. Obviously, the membrane of the bridge surface becomes incorporated into the single spherical surface resulting from the coalescence. The smoothness of the resulting sphere indicates that the bridge membrane is continuous and identical in nature with the cell membranes. This behavior is in full accord with the previously cited properties of the membrane and its concept as an elastic surface.

The tensile stress  $\sigma_s$  (force per unit of length) in the membrane of a spherical cell of radius  $r_s$  (see fig. 12) having a positive pressure differential of  $\Delta p_i$  across

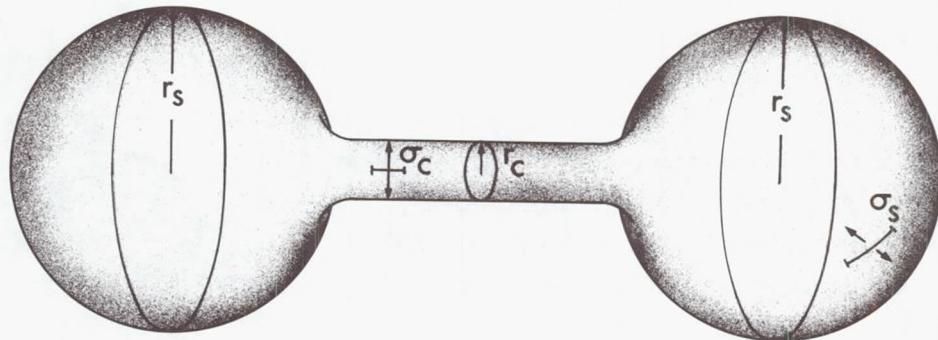


Figure 12.- Schematic illustration of dimensional definitions used in coalescence stress analysis.

the membrane ( $\Delta p_i$  = Interior static pressure minus exterior static pressure) is given by

$$\sigma_s = \frac{1}{2} \Delta p_i r_s \quad (1)$$

The stress  $\sigma_c$  in the membrane of a cylindrical bridge of radius  $r_c$  under the same pressure differential is given by

$$\sigma_c = \Delta p_i r_c \quad (2)$$

Consequently, for all values of  $r_s$  greater than twice  $r_c$ , the stress is greater in the cell membrane. In all cases,  $r_s \gg r_c$  prior to any appreciable swelling of a cell pair. Thus, when  $\Delta p_i$  increases as a result of the osmotic influx of water into the cells and bridge during hypotonic perfusion, the stress at equilibrium in the cell membrane is many times that in the bridge membrane and the cell surface is therefore stretched to a much greater degree. Since the cell membrane has an extremely small modulus of elasticity, the stretch in the cell membrane is large for even minute pressure differences.

Swelling of the cell proper results in a continuous enlargement of the adjacent portion of the bridge and a continuous shortening of the bridge as more of its membrane becomes incorporated at each end into the spherical surfaces of the cells. Ultimately, the bridge reaches zero length and becomes highly unstable, and upon further swelling of the cells, the final constriction expands so that all parts of the membrane assume the same stress.

#### Other Examples of Intercellular Bridges and Coalescence

Fawcett, Ito, and Slautterback (ref. 7) have demonstrated by electron microscopy and by phase-contrast observations the existence of intercellular bridges of the type considered in this report in spermiogenesis in a wide variety of animal forms ranging from hydra to man. Such bridges were also found to constitute a basic feature of nematocyst formation in the hydra. In both spermiogenesis and nematocyst formation, chains of bridge-connected cells evolving from the sequential divisions of an initial blast cell and its progeny were formed. The complex differentiation ontogeny of the member cells of the resulting syncytium was found to be precisely synchronized, ostensibly by means of the intercellular communication afforded by the bridge system. Electron microscopy showed clearly that the bridges were fully open cytoplasmic channels surrounded by a continuous surface membrane which was integral with the cell surface membrane. The bridges were observed to form as a result of incomplete cytokinesis which was due to the failure of the mitotic spindle to dissolve fully until long after telophase. This initial spindle remnant served to stabilize the bridge immediately after formation, and following dissolution of the remnant, the bridge membrane itself appeared to assume the role of

stabilization. This bridge-formation ontogeny by incomplete cytokinesis is precisely analogous to that observed in tissue culture with the L-strain fibroblasts of the present report.

Fawcett and others also observed the spontaneous coalescence of spermatids, again precisely analogous in its basic ontogeny to the osmotically induced coalescence of L-cells described in the present paper. Within the testis, bridges in connected spermatids were found to be quite stable, even though continual divisions of the syncytium were occurring, possibly because the individual cells were held rather firmly in the folds of the associated Sertoli cells. In freshly macerated tissue, however, it was observed that spermatid chains which had become dislodged from the Sertoli cell surface were unstable and soon spontaneously coalesced into single multinucleate giant cells. Whether any degree of swelling of the spermatids was observed to accompany the onset of coalescence was not stated.

#### Effect of Molecular Size in Bridge-Mediated Communication

Loewenstein (ref. 8) has shown recently the existence (in epithelial tissues of vertebrates and insects and in mammalian liver) of rather extensive intercellular communication via a mechanism somewhat different from the tubular bridges discussed previously. It was found that long chains of cells in these tissues conduct ionic currents as if there were no intervening membrane, the total electrical resistance being essentially the same as for cytoplasm alone. It was also found that relatively large molecules (with molecular weights of at least  $70 \times 10^3$  but less than  $127 \times 10^3$ ) could diffuse readily along such chains, but not laterally outward from the chain. Electron microscopy revealed the presence of large areas of closely applied membrane surfaces of adjacent cells, which were termed "septate junctions."

Although the ultrastructure of these junctions has not been elucidated, a plausible (but purely hypothetical) morphological interpretation which agrees generally with the electron micrographs and electrical and diffusional characteristics established by Loewenstein (ref. 8) is given in figure 13(a). The junction is presumed to consist of two closely applied unit membranes which are regularly permeated by open, closely spaced pores formed by a continuation of each membrane (or a modification thereof) to form the walls of the pore. In this scheme, the membranes of adjacent cells form one continuous surface and the cytoplasm is fully continuous, precisely as in a cell pair connected by a single intercellular bridge. In effect, in this conception of the septate junction there is a very large number of tiny intercellular bridges, the situation being analogous to a single large bridge containing a (molecular) sieve plate at the junction. For chains of cells joined in such a manner, the membrane is actually one continuous surface and the cell chain might be defined as a semi- or pseudosyncytium. This picture of the septate

junction serves, at least as a working hypothesis, to explain the low electrical and diffusional resistances in the axial direction of the cell chain and the high resistances in the lateral direction even at the cell junctions, as well as the size limit measured for molecular diffusion. On the basis of Loewenstein's electron micrographs, the areas corresponding to what have been called pores herein suggest an opening some 70 to 100 angstroms in diameter.

By use of the same analogy between the membrane dynamics in phagocytosis and in the fusion of cell pseudopods to form intercellular bridges as discussed in reference 1, it is not difficult to picture how cell junctions with the structure just discussed could arise, at least in a theoretical sense. As illustrated in figure 13(b), contacts of microporjections on one or both cell surfaces, formed initially perhaps as the result of a specific attractive molecular mosaic on the cell surface, could fuse to produce the microchannels.

Regardless of the fine structural details of septate junctions, however, it should be noted that in regard to intercellular communication there is a fundamental difference between this junction form and the simple "single-channel" bridge. Although the septate junction may have a very large total area of cytoplasmic continuity, and hence allow efficient transport of small ions and molecules, a small effective pore size can serve to block interchange of all larger molecular species. Intercellular communication in this case is therefore subject to specific regulation. In the case of the simple single-channel bridge, however, even though the total maximum cross-sectional area of cytoplasmic

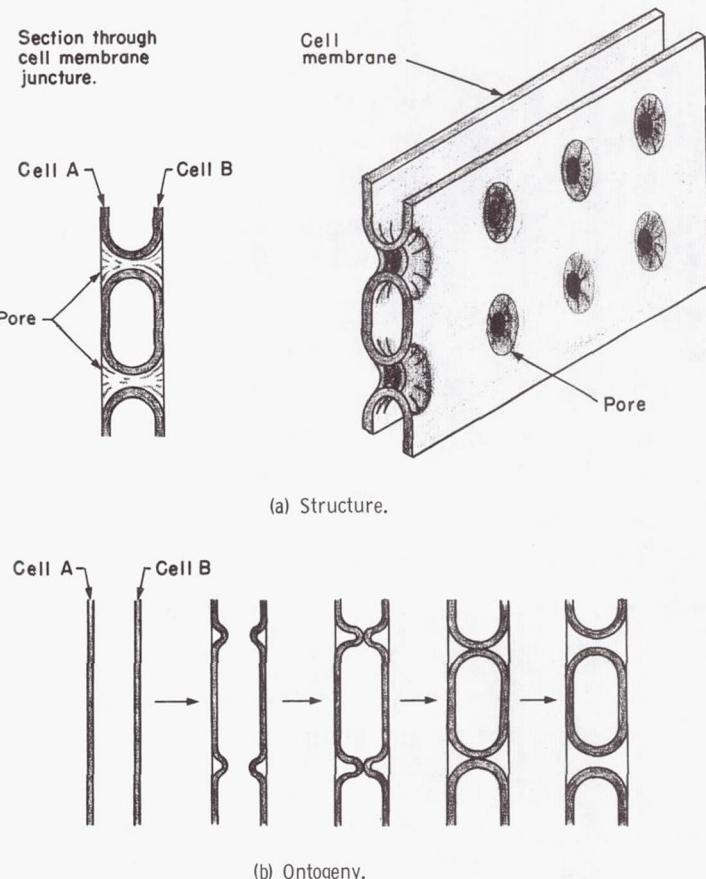


Figure 13.- Illustration of hypothetical structure and formation ontogeny of septate junction between cells *in vivo*. See text for discussion of details.

continuity may be even much smaller than that for the junction, the total sectional area is continuous and hence permits transport of all molecular species and perhaps even cellular organelles<sup>2</sup>.

### COALESCENCE INVOLVEMENT IN GIANT-CELL FORMATION

Spontaneous coalescence of bridge-connected cells in tissue culture to form mono- or binucleate giant cells is frequently observed in time-lapse films of malignant cell cultures such as the L-strain and HeLa lines following telophase (refs. 1 and 9) and has been termed "teloreduplication" by Moorhead and Hsu (ref. 9). Coalescence appears after telophase following bridge formation by incomplete cytokinesis and is precipitated by violent blebbing and contortions of the cell membrane while the pair is still in the rounded state. The cells ultimately fuse through the bridge, and cytoplasmic fusion is often accompanied by nuclear fusion. Subsequent attempts of these resultant giant cells to divide are often abortive, the cells failing even to attempt cytokinesis. The resultant cell of this mitosis attempt contains either a single macronucleus or several nuclei of various sizes.

The presence of appreciable numbers of mono- and multinucleate giant cells is a characteristic feature of most malignant tumors. In view of the apparent association of intercellular bridges with malignant cells in vitro (refs. 1 and 10), the possibility exists that the giant cells observed in tumors are a product of coalescence; the presence of intercellular bridges in malignant cell populations in vivo is thus suggested.

### OTHER CYTOLOGICAL APPLICATIONS

Although the primary concern of this paper has been with a description of osmotic coalescence techniques and implications of the coalescence phenomenon for cytoplasmic continuity and molecular communication in malignant cell syncytia in vitro, the osmotic swelling technique has several other proved or potential cytological applications of interest, a few of which are mentioned briefly in the following sections.

#### Giant Cell Production

Coalescence results in the production of giant cells, and provided proper care is exercised in the treatment process, it is possible to obtain these cells in a viable state for use in subsequent studies. Such cells, when formed by coalescence of cells whose

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<sup>2</sup>Fawcett, et al. (ref. 7) have observed continuous sheets of endoplasmic reticulum extending through intercellular bridges of spermatids.

histories have been established by time-lapse recordings, constitute valuable specimens for cytogenetic studies.

#### Formation of Karyotypic Hybrids

The spontaneous formation of karyotypic hybrid cells in mixed cultures is well documented, apparently occurring by fusion of both cytoplasm and nuclei of the reacting cells. It is of interest to note that in most of these "crosses," at least one of the reactants was malignant in character and demonstrated bridge formation *in vitro*. It is possible that hybridization in such cells occurs by means of external formation of intercellular bridges by pseudopod contact (ref. 1) followed by coalescence. If such is the case and an appreciable number of such bridges exist at any one time, osmotic swelling should appreciably increase the frequency of hybrid formation in mixed cultures. Additionally, it should be possible to determine conclusively, by the use of time-lapse recording and osmotic swelling, if malignant cells are capable of forming by external pseudopod merger true intercellular bridges with normal cells. Osmotic coalescence of such a cell pair would yield a valuable cell system for cytogenetic study. The microlagoon technique of references 11 and 12 offers an excellent means for isolating and confining cell pairs or small groups of cells for such studies.

#### Molecular and Ionic Infusion

The well-known phenomenon of hemolysis, in which red blood cells cease to expand without rupture upon reaching a large volume in hypotonic media, is an example of membrane permeability increase in response to stretching. The gross stretching of the membrane so increases its permeability that the large hemoglobin molecules escape in great numbers and thus apparently reduce the osmolarity of the cell contents to a value which precludes further swelling. The same process of osmotic swelling can be used to accomplish the reverse situation, that of putting into a cell ions or molecules which normally would not permeate the membrane. By using a perfusion solution which is hypotonic in overall concentration, but hypertonic in some ion or molecule whose infusion is desired, osmotic swelling to a sufficient degree can often result in appreciable infusion.

#### Local Swelling of Membrane Regions

It is often desirable to treat or examine small regions of cells without altering or disrupting the entire system. Using very fine micropipets, it is possible by highly localized hypotonic perfusion to distend small areas of a cell. This technique has been found useful for such purposes as breaking surface attachments, determining membrane continuity, increasing membrane permeability for local infusion or exfusion, generating structural stresses, and the like.

## CONCLUDING REMARKS

The subject of intercellular bridges is one of considerable practical importance, especially in view of the indications that mitotic stimuli can be transmitted via these connections. Pairs of bridge-connected cells offer an excellent system for investigation of the mechanisms of mitosis initiation, since molecular transport through the bridge from a dividing cell to an interphase cell can potentially be monitored by cytochemical techniques. Such systems also offer a valuable means for studies of radiation-induced suppression of mitosis, since exchange of specific biochemical products resulting from intercellular molecular communication between a normal and an irradiated cell can possibly be monitored by means of the bridge connection. The osmotically induced coalescence techniques outlined in this paper provide a simple and direct method for establishing whether an apparent bridge between cells in vitro is truly cytoplasmically continuous, and hence these techniques should be of particular value in investigations involving bridge-connected systems and similar syncytia.

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